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HYDROPHOBIC INTERACTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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SUMMARY

Protein separations by means of a new hydrophobic-interaction high-performance liquid chromatography column are described. A linear elution gradient was used, starting with 1.7 *M* ammonium sulfate and concluding with 0.1 *M* sodium phosphate (pH 7.0). All proteins except albumin and β-lactoglobulin were eluted as sharp peaks. The broadening of some protein peaks may depend upon the influence of the eluent on the structure of those proteins. Variations in column temperature and additions of either methanol or urea to the mobile phase were studied. The results show that small adjustments in chromatographic conditions can greatly improve resolution when band spreading exists. In addition, this column can be used not only for the separation of proteins, but also to determine their relative hydrophobic character.

INTRODUCTION

Hydrophobic-interaction chromatography was developed after variations in retentive behavior of samples were observed in affinity chromatography[1–10]. These differences in retention were dependent upon the length of the hydrophobic spacer arm that holds the functional group on the matrix. Consequently, proteins could be separated by differences in their hydrophobic character. Other conditions regarding protein chromatography on hydrophobic packings have been discussed[11–15].

Hydrophobicity is defined here as the strength of interaction between a protein and a non-polar ligand, in this case a phenyl group. Using this definition, more "hydrophobic" proteins are not necessarily more soluble in nonpolar solvents. Instead, hydrophobicity is used in relation to proteins that are soluble in an aqueous phase.

Two classes of high-performance liquid chromatographic (HPLC) techniques are currently designed to separate compounds according to their hydrophobic behavior. These are reversed-phase and hydrophobic-interaction chromatography. In both cases, hydrophobic functional groups (aliphatic or aromatic) can be bound to the substrate and used as the functioning stationary phase. The most outstanding physical difference between hydrophobic and reversed-phase matrices is in the density of exposed hydrophobic groups. Reversed-phase packings commonly have ten to one

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hundred times the density of hydrophobic groups in comparison with hydrophobic-interaction chromatography packings. As a result, proteins are eluted using less severe conditions in hydrophobic-interaction chromatography than in reversed-phase chromatography.

Until recently, hydrophobic packing materials for hydrophobic-interaction chromatography were not readily available for HPLC. During development work on the Bio-Gel TSK Phenyl 5-PW column, a similar hydrophobic-interaction HPLC column was described which utilized silica as the support matrix[15]. Since silica will dissolve under alkaline conditions, this was a severe limitation for hydrophobic interaction chromatography. An improved design includes the use of hydroxylated polyether as a support material. The present study explores some characteristics of protein separations on this phenyl hydrophobic-interaction HPLC column, which is now commercially available.

EXPERIMENTAL

All reagents were of the highest purity available. HPLC-grade ammonium sulfate was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Proteins were purchased from Sigma (St. Louis, MO, U.S.A.). All chromatograms were obtained with the Bio-Rad HPLC Protein Analysis System, a Model 1305A variable-wavelength UV monitor and a Model 1322 dual-pen strip chart recorder. Gradients were monitored with a Bio-Rad conductivity monitor and a standard flow cell. Phenyl Sepharose was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). The Bio-Gel TSK Phenyl-5PW column was from Bio-Rad. All buffers for either open column chromatography or HPLC were passed through 0.45-μm filters. The HPLC column temperature was regulated by immersing it in a Lauda RC20 water bath (Brinkman Instruments, Westbury, NY, U.S.A.).

RESULTS

HPLC separation of individual proteins

The proteins that were studied (Table I) ranged in molecular weights from 12,400 (cytochrome *c*) to 68,500 daltons (serum albumin). These proteins are listed in Table I in the order of their relative retention on the Bio-Gel TSK Phenyl-5PW column when a linear ammonium sulfate gradient was used. This 60-min gradient at room temperature started with 1.7 *M* ammonium sulfate and 0.1 *M* sodium phosphate buffer at pH 7.0 and ended with an otherwise identical buffer but without ammonium sulfate. Proteins were injected and studied individually. The most strongly retained proteins are presumed to be the most hydrophobic and are at the bottom of Table I. An unretained protein such as cytochrome *c* has a relative hydrophobicity of 0, because it is eluted with the eluent front. The most strongly retained protein is chymotrypsin, which was eluted after 0.82 h in the 60-min gradient. The relative hydrophobicity of proteins appears to be unrelated to their molecular weight. It is not clear from this table whether the hydrophobic retention parameter derived from the elution time (Table I) is actually related to their native non-polarity. Instead, the values given in Table I provide a reference point for purposes of comparison.

TABLE I

PROTEINS STUDIED WITH THE BIO-GEL TSK PHENYL 5PW COLUMN

Proteins were prepared in 1.7 *M* ammonium sulfate, 0.1 *M* sodium phosphate (pH 7.0), applied to the Bio-Gel TSK Phenyl-5PW column, and eluted with a 60-min linear gradient, starting with 1.7 *M* ammonium sulfate, 0.1 *M* sodium phosphate (pH 7.0) and ending with 0.1 *M* sodium phosphate (pH 7.0). Relative hydrophobicity was determined from the normalized retention time under these gradient conditions. A value of 1.0 indicates elution at the end of the 60-min gradient.

Protein	*Molecular weight (daltons)*	*Relative hydrophobicity from retention times*
Cytochrome *c*	12,400	0.0
Myoglobin (horse heart)	16,900	0.23
Ribonuclease A	12,600	0.35
Ovalbumin	44,000	0.51
β-Lactoglobulin	37,100	0.58
Serum albumin (bovine)	68,500	0.59
Hemoglobin	64,500	0.64
Lysozyme	13,900	0.65
Chymotrypsin	22,600	0.82

Comparison of open-column and high-performance liquid chromatography

Of the proteins in Table I, only a few were chosen for additional analyses. These were selected on the basis of their elution characteristics under the chromatographic conditions used (Table I). Three of the four proteins that were most intensively investigated (cytochrome *c*, myoglobin and lysozyme) have similar molecular weights (Table I). Fig. 1 shows a separation of cytochrome *c*, lysozyme and myoglobin on a hydrophobic phenyl agarose matrix by gravity flow. All three proteins were separated easily under these conditions.

The separation achieved in Fig. 1 was repeated under similar conditions on the analagous Bio-Gel TSK Phenyl-5PW HPLC column (Fig. 2A). Here, an otherwise similar chromatogram was developed faster and gave much higher resolution than that shown in Fig. 1.

Albumin and peak broadening

Under the conditions defined in Table I, both albumin and β-lactoglobin were eluted from the Bio-Gel TSK Phenyl-5PW column as broad peaks. Albumin was chosen for further analyses to aid in understanding this phenomenon. Fig. 2B shows a typical chromatogram of bovine serum albumin at 25°C. This chromatogram can be divided into at least three components: contaminants, which appear as small peaks and are eluted at retention times less than *ca.* 17 min, a sharp shoulder at *ca.* 19 min, and a broad peak, centered around 21 min. The shoulder cannot be caused by contaminants, because when it was collected and rechromatographed under identical conditions, both the shoulder and the broad peak reappeared as in Fig. 2B. When other fractions of the broad albumin peak were rechromatographed, they were also eluted as in Fig. 2B.

The elution profile of serum albumin was compared with that of defatted serum albumin (Fig. 3). The chromatogram of fat-free albumin (Fig. 3B) showed fewer contaminants (at retention times below *ca.* 18 min), but gave the same broad peak

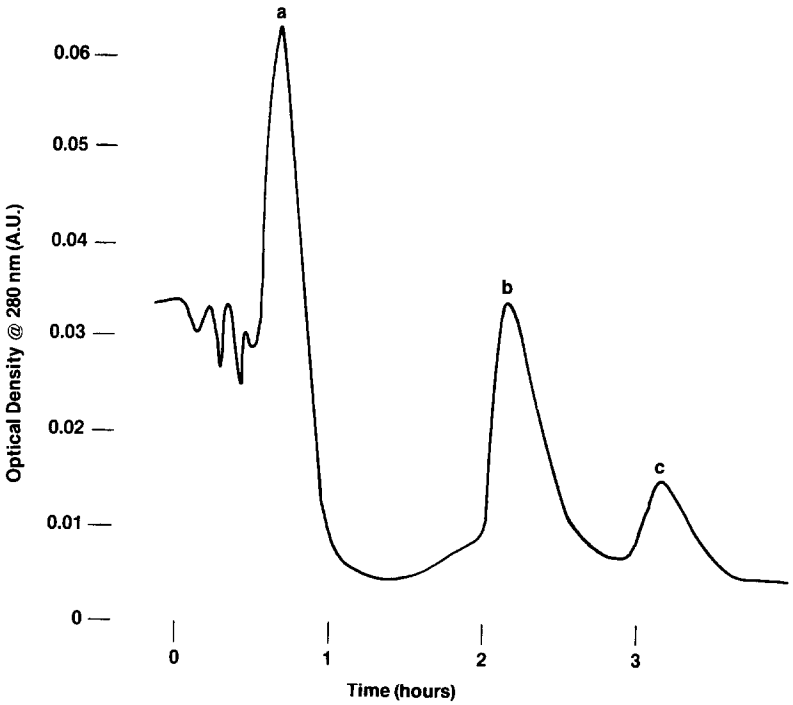

Fig. 1. Open-column chromatogram of a mixture of proteins. a = Cytochrome *c* (0.7 mg); b = myoglobin (0.7 mg); c = lysozyme (0.2 mg). This mixture was injected in 0.5 ml. A linear gradient was applied from 1.7 *M* ammonium sulfate, 0.1 *M* sodium phosphate (pH 7.0) to 0.1 *M* sodium phosphate (pH 7.0) in 3.5 h by gravity flow. The 1.5-cm diameter glass column was packed with 10 cm of Phenyl Sepharose.

(at *ca.* 25 min) as serum albumin which had not been defatted (Fig. 3A). The small difference in the height of the shoulder between these two samples was not significant and will be discussed later.

Temperature effects

The retention behavior of cytochrome *c*, myoglobin, lysozyme and albumin was studied as a function of temperature. In each case, the stainless-steel column and adjacent tubing were immersed in a temperature-controlled water bath. A series of chromatograms were obtained at 5 ± 0.02°C intervals from 0 to 45°C, and retention times were determined.

Cytochrome *c* remained at the solvent front below 30°C. Above 30°C, there was a non-linear but sharply increasing retention time of this protein up to 45°C. The peak associated with the elution of cytochrome *c* remained narrow within both the upper (40 and 45°C) and lower (0–25°C) temperature ranges, but at 30 and 35°C, this protein peak appeared short and broad. The broad peak is assumed to be due to weak retention of the cytochrome *c* in the starting buffer and subsequent (nearly) isocratic elution. When the cytochrome *c* was more strongly retained, elution required a gradient and, as expected, the peak became sharper. The retention behavior of myoglobin was similar to that of cytochrome *c* in that it was retained longer at higher

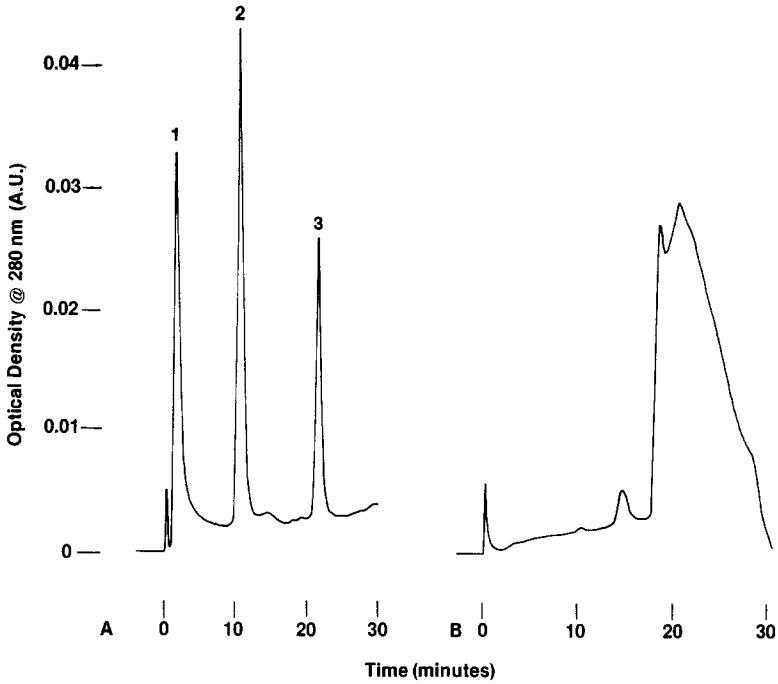

Fig. 2. Chromatograms of selected proteins on the Bio-Gel TSK Phenyl-5PW column at 25°C. A, Cytochrome *c* (1), myoglobin (2) and lysozyme (3) were injected as a mixture (20 μl of the mixture described in Fig. 1). B, Only bovine serum albumin was injected (20 μl of a 25 mg/ml solution). In both chromatograms, a 30-min linear gradient from 1.7 *M* to 0 *M* ammonium sulfate was used, as in Table I.

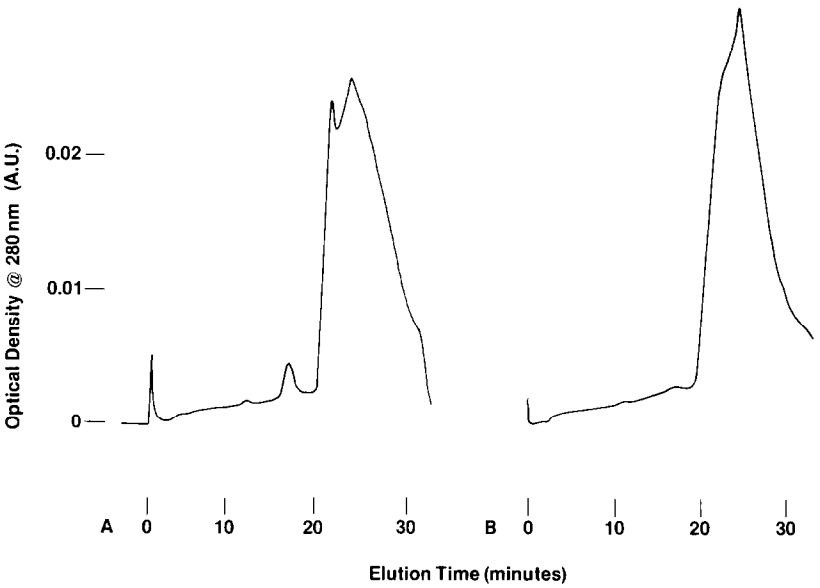

Fig. 3. Chromatograms of albumin (A) and defatted albumin (B). Conditions were as in Fig. 2. Little difference in elution profile was seen between the two kinds of albumin at this or other temperatures.

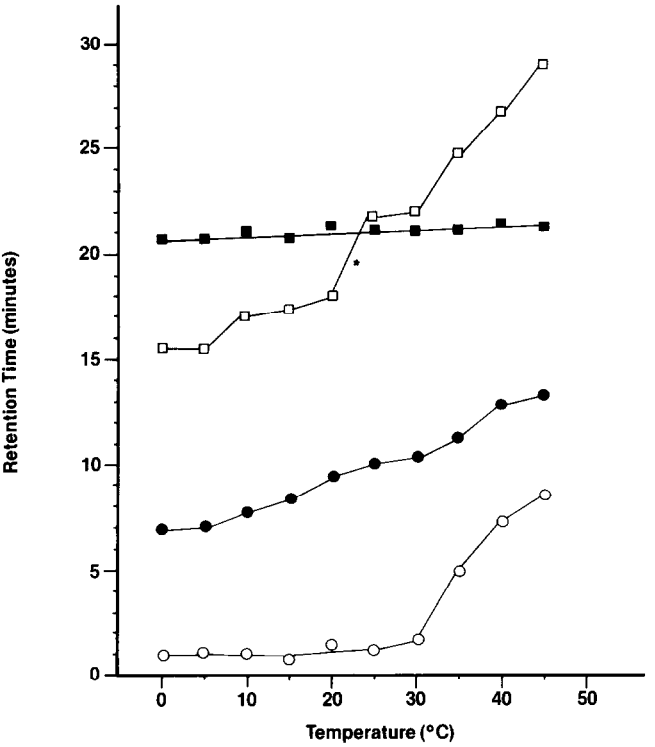

Fig. 4. Influence of temperature on retention behavior of various proteins. The asterisk (★) indicates where the broad and sharp albumin peaks were the same height in the chromatogram. Above the asterisk the broad peak was tallest, and below the sharp peak was tallest. Retention time was taken from the peak with the maximum absorbance. Elution conditions were identical to those in Fig. 2, except that the column was immersed in a water bath temperature-controlled to ±0.2°C. The proteins that were analyzed included cytochrome *c* (○), myoglobin (●), bovine serum albumin (□), and lysozyme (■).

temperatures (Fig. 4). However, unlike cytochrome *c*, myoglobin was never eluted with the eluent front in the 0–40°C range. Under these conditions, myoglobin always behaved ideally, being eluted as a sharp band.

The two proteins that gave the most unusual retention behavior were lysozyme and albumin. Lysozyme was unusual because it was the only protein which demonstrated essentially no change in either profile or retention time with temperature. Albumin, on the other hand, not only showed a non-linear increase in retention time with temperature but its elution profile also changed dramatically from 0 to 30°C. Above 30°C, albumin was eluted in a single, broad peak. As the temperature was lowered below 25°C, a sharp peak that was not completely resolved from the main (broad) peak, appeared at *ca.* 21 min. As the temperature was decreased further, the sharp peak increased in height and the broad peak became smaller (Fig. 3A). The conversion between the broad and sharp peaks appeared to be continuous over the range 0–30°C. At 0°C, the broad peak could not be distinguished from the baseline, while above this temperature the broad peak became significant. Therefore, small differences in temperature could cause large variations in the elution profile of al-

bumin near 25°C. The differences observed in Fig. 3A and B are likely to be due to small shifts in the laboratory temperature during analyses.

Effects of methanol and urea

Solvents that are compatible with this column have been described[16]. In an attempt to test different types of buffer components which would enhance desorption of proteins, both an organic solvent (methanol) and a chaotrope (urea) were studied. In each case, the concentration of the additive was kept constant during a salt gradient which was otherwise similar to that described for Fig. 2. Fig. 5 shows the effect of adding 5 or 10% methanol to both solvents on the retention behavior of cytochrome *c*, myoglobin, lysozyme and albumin. The addition of methanol had an effect on retention behavior similar to that obtained when the temperature was decreased (Figs. 4 and 5). In addition, both lowering the temperature and adding methanol caused the broad albumin peak to become very sharp (Fig. 6).

Methanol (either 5 or 10%) also reduced retention times of cytochrome *c*, myoglobin and albumin. However, lysozyme was eluted with an increased retention time when 5% methanol was added, and it was eluted with a shorter retention time with 10% methanol (Fig. 5). All these proteins behaved ideally at both methanol concentrations in that they gave sharp elution profiles.

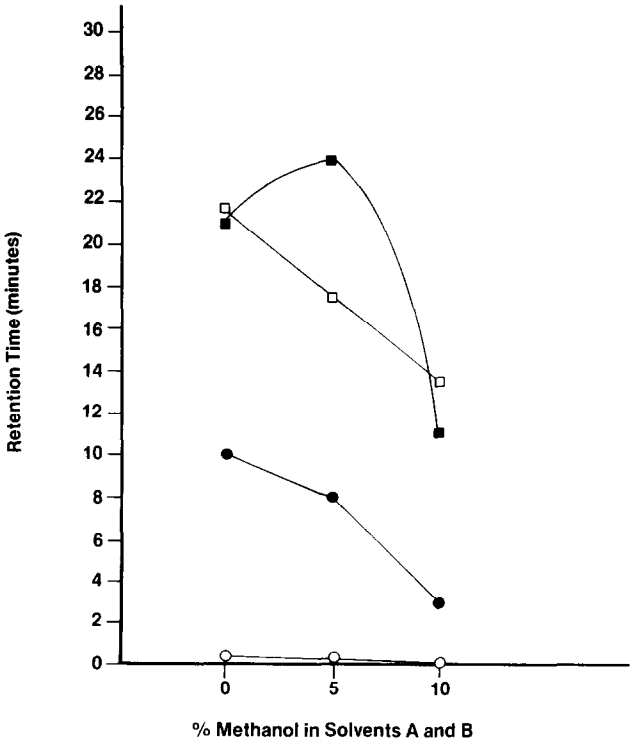

Fig. 5. Effect of methanol on the retention behavior of various proteins. Conditions were the same as in Fig. 2, except buffers A and B contained either 5 or 10% methanol. Proteins: cytochrome *c* (○); myoglobin (●); bovine serum albumin (□); lysozyme (■).

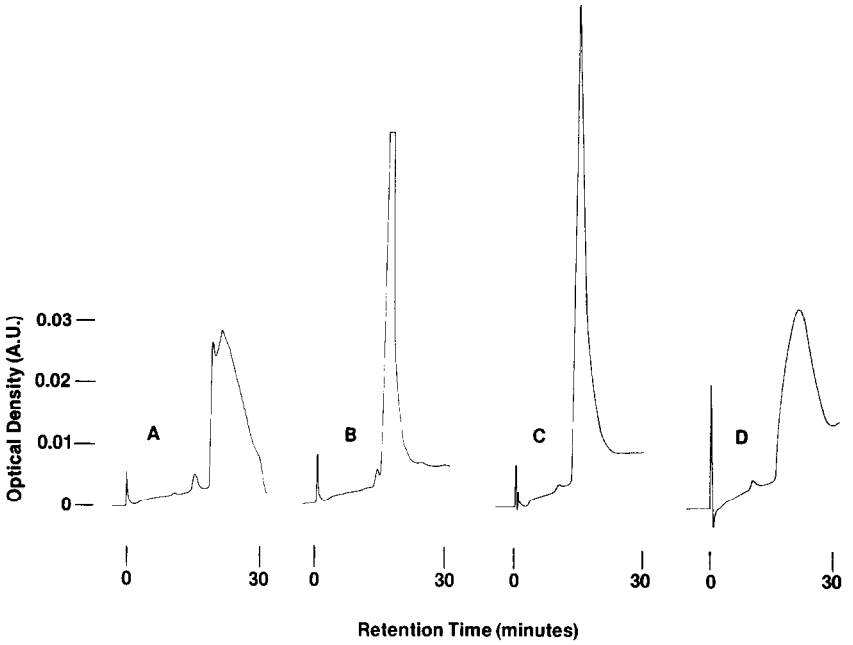

Fig. 6. Effect of temperature, methanol, and urea on the elution profile of bovine serum albumin. General conditions are described in Figs. 3, 5, and 7. Specifically, albumin was analyzed at 25°C (A) and 0°C (B) or at 25°C with 10% methanol (C) or 4 *M* urea (D) in both eluents.

The influence of urea on protein retention times is shown in Fig. 7. Urea generally reduced retention times of the proteins. However, the effects were different from both temperature (Fig. 4) and methanol (Fig. 5) effects. The retention times for lysozyme diminished continuously as the concentration of urea increased (Fig. 7). This was not the case with either temperature (Fig. 4) or methanol (Fig. 5). Both albumin and fat-free albumin showed little change in retention time or elution profile with the addition of urea (Figs. 6 and 7).

DISCUSSION

Most of the information obtained in this study demonstrates expected relationships between mobile phase, stationary phase and proteins in high-performance hydrophobic-interaction chromatography. Decreasing the temperature or adding either organic solvent or urea to the eluent reduced the strength of the hydrophobic interaction and accelerated elution of the applied proteins. Lysozyme was an exception in that its retention behavior was not influenced by temperature and low concentrations of methanol slightly increased its retention time. No attempt was made to investigate the reasons for this unusual behavior, since lysozyme was otherwise eluted in a single, sharp peak.

The Bio-Gel TSK Phenyl-5PW column was chosen for this investigation because of the commercial availability of both this hydrophobic column and a comparable open-column packing material. The similarities between chromatograms ob-

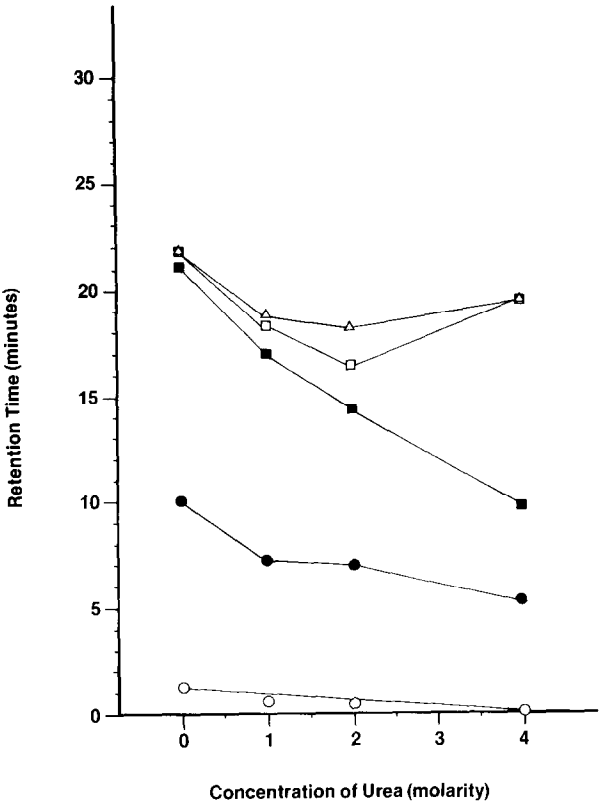

Fig. 7. Effect of urea on retention behavior of various proteins. Proteins were chromatographed under conditions similar to those described in Fig. 2, except that either 0, 1, 2, or 4 *M* urea was added to both eluents. Proteins: cytochrome *c* (○); myoglobin (●); lysozyme (■); bovine serum albumin (□); fat-free bovine serum albumin (△).

tained with the open-column material (Fig. 1) and the HPLC column (Fig. 2A) suggest that the elution order of sample components will be essentially the same with Phenyl Sepharose and Bio-Gel TSK Phenyl-5PW.

The strength of the hydrophobic interaction between two molecules generally increases with temperature[17]. In hydrophobic-interaction chromatography, this effect usually necessitates a lower salt concentration for desorption of proteins at higher temperatures, as shown in Fig. 4. Even though reversed-phase separations also depend upon the hydrophobic effect, retention times of samples on these columns generally decrease with temperature[18–20]. This apparent discrepancy is probably due to the influence of organic solvents. On the Bio-Gel TSK Phenyl-5PW column, the retention times of all four proteins were generally reduced with increased temperature (Fig. 4), as would be expected if solvent effects did not interfere. However, the influence of temperature on the hydrophobic effect was not the same for all the proteins, since their retention times were influenced differently (Fig. 3).

The retention time of albumin was influenced as expected by elution conditions. However, its elution profile was ideal only under special circumstances. It is

not clear from the present study why albumin was eluted in a sharp band by high concentrations of ammonium sulfate, and a broad band when it was eluted by lower salt concentrations. This behavior could possibly be caused by a change in protein conformation induced by ammonium sulfate. Regardless of the mechanism responsible for altering the peak shape of albumin or other proteins, it is clear from these studies that in at least some cases, the elution profile of proteins for the Bio-Gel TSK Phenyl-5PW column can be dramatically influenced by relatively small alterations in conditions.

Lowering the temperature is in many cases the most favorable means of improving resolution for the isolation of purified enzymes or other natural products for which retaining the native structure is crucial. However, other methods are also available. These mostly include the manipulation of eluents for optimizing separations (Figs. 5 and 7).

The differences between the effects of urea and methanol on the elution of albumin demonstrates the influence various eluents can have on the sample and also that they are often dependent upon the physical characteristics of sample–eluent interactions. Urea decreased the retention time of albumin but did not show the same effect on peak shape as reducing the temperature or adding methanol. This may be because urea will tend to counteract the stabilizing effect of ammonium sulfate. In addition to its effects on albumin, urea diminished the retention times of the remaining proteins (Fig. 7). This study has been restricted to a general analysis of the manner in which a few water-soluble proteins behave on the Bio-Gel TSK Phenyl-5PW HPLC column. This column separates proteins by a unique adsorption–desorption mechanism. The relative retention times and retention behavior of different proteins are a function of their affinity for a hydrophobic ligand. In reversed-phase chromatography, many proteins are eluted with high concentrations of organic solvent, partly because the solvent-denatured conformation binds more tightly than the native conformation. When eluted from the Bio-Gel TSK Phenyl-5PW column, many enzymes retain their activity, and the high recoveries[16] suggest that denaturation is minimal.

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